Biological characterisation of *Sarcocystis neurona* isolated from a Southern sea otter (*Enhydra lutris nereis)*

D.S. Lindsay[a,*], N.J. Thomas[b], J.P. Dubey[c]

[a]*Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, VA 24061-0342, USA*
[b]*Department of the Interior, United States Geological Survey, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711, USA*
[c]*United States Department of Agriculture, Agricultural Research Service, Livestock and Poultry Sciences Institute, Parasite Biology and Epidemiology Laboratory, BARC-East, Beltsville, Maryland 20705-2350, USA*

Abstract

Sarcocystis neurona was isolated from the brain of a juvenile, male southern sea otter (*Enhydra lutris nereis*) suffering from CNS disease. Schizonts and merozoites in tissue sections of the otter's brain reacted with anti-*S. neurona* antiserum immunohistochemically. Development in cell culture was by endopolyogeny and mature schizonts were first observed at 3 days postinoculation. PCR of merozoite DNA using primer pairs JNB33/JNB54 and restriction enzyme digestion of the 1100 bp product with *Dra* I indicated the organism was *S. neurona*. Four of four interferon-γ gene knockout mice inoculated with merozoites developed *S. neurona*-associated encephalitis. Antibodies to *S. neurona* but not *Sarcocystis falcatula*, *Toxoplasma gondii*, or *Neospora caninum* were present in the serum of inoculated mice. This is the first isolation of *S. neurona* from the brain of a non-equine host. © 2000 Published by Elsevier Science Ltd on behalf of the Australian Society for Parasitology Inc. All rights reserved.

Keywords: Encephalitis; *Enhdyra lutris nereis*; *Sarcocystis neurona*; Southern sea otter

1. Introduction

Equine protozoal myeloencephalitis is a neurological condition seen in horses in the Americas and it is caused by *Sarcocystis neurona* [1]. Clinical signs of the disease are caused by schizonts in the CNS. No sarcocysts are present. Horses are accidental hosts, the natural intermediate host is not known. This parasite is transmitted in the faeces of opossums (*Didelphis virginiana*) [2]. Although similar cases of encephalitis associated with *S. neurona*-like parasites have been reported in raccoons (*Procyon lotor*) [3], mink (*Mustela vison*) [4], a striped skunk (*Mephitis mephitis*) [5],

a rhesus monkey (*Macaca mulatta*) [6], Pacific harbor seals (*Phoca vitulina richardsi*) [7], and a sea otter (*Enhdyra lutris*) [8], these studies have been unable to isolate the parasite for definitive identification

The present report documents the isolation of *S. neurona* from a southern sea otter (*Enhydra lutris nereis*), its development in cell culture, and transmission to immunodeficient mice. This is the first time this parasite has been isolated from the brain of a non-equine intermediate host.

2. Materials and methods

2.1. Case history

Since 1992, researchers at the National Wildlife

---

* Corresponding author. Tel.: +1-540-231-6302; fax: +1-540-231-3426.

E-mail address: lindsayd@vt.edu (D.S. Lindsay).

Health Center, Madison, WI, have been conducting necropsy examinations on southern sea otters as part of a mortality monitoring program to define factors which may be responsible for limiting the growth of the southern sea otter population. We are especially interested in cases of encephalitis in these animals [9].

A juvenile male southern sea otter was found hauled out on a public beach at the northern aspect of Monterey Bay, CA, shivering and minimally responsive. Attempts to return the animal to the water resulted in its return to the beach. At that point, the otter was captured without incident by volunteers from the Marine Mammal Center and brought to the Monterey Bay Aquarium for medical evaluation and subsequent rehabilitation.

At the time of presentation the otter was estimated to be approximately 24 weeks of age based upon its full complement of adult teeth lacking significant wear, full adult pelage, body length (101 cm), and body girth (41.5 cm). Arrival examination revealed a stuporous otter, moderately emaciated (wt = 9.2 kg), with a rectal temperature of 31.4°C (normal = 37.5–38.1°C). The pulse and respiratory rates were normal. Mucous membranes were somewhat hyperaemic. The pelage was matted. Despite the efforts of staff veterinarians to save the otter, it was found dead on day 10. The carcass was placed in a freezer for 4 h to facilitate cool down, and then refrigerated and sent by overnight express service to the National Wildlife Health Center in Madison.

At the National Wildlife Health Center, one-half of the brain was fixed for light microscopy by immersion in 10% buffered formalin. Additional formalin fixed tissue samples included the upper cervical spinal cord, heart, tongue, skeletal muscle, lung, liver, kidney, urinary bladder, pituitary gland, adrenal gland, thyroid gland, spleen, lymph node, thymus, pancreas, and intestine. Formalin-fixed tissues were routinely processed for paraffin embedding, sectioned at 5 μm, and stained with H&E. For immunohistochemical staining, paraffin sections of brain were reacted with rabbit anti-*S. neurona*, *Toxoplasma gondii* or *Neospora caninum* antibodies as described [2,10].

2.2. In vitro isolation and development of Sarcocystis neurona

An approximately 20 g portion of brain was sent by overnight courier to the Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, for parasite isolation. The brain tissue was homogenised in 20 ml of Hanks balanced salt solution without calcium and magnesium (HBSS) and a 10 ml portion of the homogenate was inoculated on to African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection) and bovine turbinate cells (BT cells, ATTC CRL 1390, American Type Culture Collection). After 30 min the homogenate was removed and fresh culture medium was added. Merozoites were maintained in both CV-1 and BT cell cultures. The host cells were grown in plastic cell culture flasks in growth media consisting of 10% (v/v) foetal bovine serum in RPMI 1640 medium supplemented with 100 U penicillin G/ml, and 100 mg streptomycin/ml. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

For descriptive studies and mouse inoculations, merozoites were harvested from infected cell cultures by removing the medium and replacing it with HBSS. The host cells were then removed from the plastic growth surface by use of a cell scraper. This cell mixture was passed through a 27-gauge needle attached to a 10-ml syringe to rupture host cells. The suspension was then filtered through a sterile 3 μm filter to remove cellular debris. The numbers of merozoites in the filtrate was determined using a haemocytometer.

The developmental cycle in vitro was examined in BT and CV-1 host cells. The merozoites were inoculated on to 22 mm^2 glass coverslips containing a monolayer of CV-1 or BT cells in six-welled tissue culture plates. Coverslips of cells to be examined 1 and 2 days p.i. were inoculated with 1×10^6 merozoites and all other coverslips of host cells were inoculated with 5×10^5 merozoites. Coverslips of infected CV-1 cells were removed on days 1, 2, 3, and 4 p.i. and coverslips containing infected BT cells were removed on days 1, 2, 3, 4, 5, and 6 p.i. and fixed in 10% phosphate buffered formalin for 30 min, placed in 100% methanol for 10 min and stained with a Giemsa type stain (Diff-Quick®, Dade Behring Inc.). Coverslips were attached to glass microscope slides with Permount®–(Fisher Scientific) and examined with light microscopy.

2.3. Transmission electron microscopy

A monolayer of BT cells in a 25 cm^2 cell culture flask was inoculated with 5×10^5 merozoites. Five days p.i. the infected monolayer was removed from the plastic growth surface with a cell scraper, placed in a 15 ml tube and pelleted by centrifugation. The cell pellet was fixed in 3% (v/v) glutaraldehyde in PBS (pH 7.4) for 3 days. Cell pellets were post-fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a series of ethanols, passed through two changes of propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences Inc.). Thin sections were stained with uranyl acetate and lead citrate and examined with a JOEL-100 CX II TEM operating at 80 kV.

2.4. PCR and restriction digest analysis (RFLP)

Merozoites were collected from infected host cells and frozen at −20°C until used. Between 0.8 and 8 × 10^7 merozoites were thawed and suspended in 1 ml PBS. They were vortexed and then centrifuged in a microfuge for 1 min. The PBS was removed and the samples were resuspended in 500 ml of sterile water. A 20 ml aliquot was taken and mixed with 200 ml of InstaGene Matrix (Bio Rad). The samples were then incubated in a 56°C water bath for 30 min. The samples were vortexed and then placed in boiling water for 8 min. The samples were vortexed and centrifuged in a microfuge for 2–3 min. A 20 ml aliquot of the supernatant was used per 50 μl PCR reaction. The remaining supernatant was stored at −20°C. Polymerase chain reaction was performed on each sample using Ready To Go PCR Beads (Amersham Pharmacia Biotech Inc.) and a Hybaid OmniGene thermocycler. Standard PCR reaction conditions were used with the following amplification parameters: 94°C, 5 min; 40 cycles of (94°C, 1 min; 50°C, 1 min 15 s; 72°C, 1 min); 72°C, 10 min. The PCR products were run on a 1% agarose gel. An 1100-bp PCR product was amplified using primers JNB33(5′-CGAACAGAGATGAG-GAAAAT-3′) and JNB54(5′-GTTGTGGTGTTGC-GTGAGTC-3′) [11]. The PCR products were digested separately with the restriction enzymes *Hinf* I or *Dra* I (Promega) and were analysed by electrophoresis on a 1% agarose gel with appropriate size markers. Merozoites of the SN6 strain of *S. neurona* [12] and merozoites of our cloned line the Cornell strain of *Sarcocystis falcatula* [13], designated SFVT-1-2, were processed similarly and used as controls.

2.5. Mouse inoculations and examinations.

Four female, 18–20 g, interferon-γ gene knockout (INF-γ-KO) mice (BALB/ c-Ifng[tm1Ts], Jackson Laboratories) were s.c. inoculated with 1 × 10^6 merozoites. Mice were sacrificed 19 days p.i. (one mouse), 21 days p.i. (one mouse) and 24 days p.i. (two mice). The two mice examined 24 days p.i. were bled from the retoorbital plexus prior to being sacrificed. The brain, eyes, and portions of the tongue, heart, skeletal muscle, lungs, liver, spleen, kidney and adrenal glands were fixed in 10% neutral buffered formalin solution and processed for histological examination by light microscopy.

Sera from the two mice sacrificed at 24 days p.i. were examined by IFAT. *Sarcocystis neurona* SN6 strain merozoites or *S. falcatula* Cornell strain merozoites were used as antigen and 3 × 10^4 merozoites were placed in each well of a 12-well IFAT slide. The merozoites were air-dried on the IFAT slides and the slides stored at −20°C until used. Sera were examined at doubling dilutions in PBS beginning at 1:25 and endpoint titrated. Test sera (30 μl) were incubated on the IFAT slides for 30 min at room temperature in a humidified box. The slides were then washed twice for 5 min in PBS. Anti-mouse IgG conjugate (30 μl) was then incubated with the test wells on the IFAT slides for 30 min at room temperature in a humidified box. The slides were then washed twice for 5 min in PBS and mounted in 90% glycerin-PBS solution. Slides were viewed using an Olympus epifluorescent microscope. Positive samples exhibited a complete merozoite surface fluorescence. Negative samples exhibited no fluorescence or only anterior end fluorescence (nonspecific). The sera were also examined for agglutinating antibodies to *T. gondii* [14] and *N. caninum* [15].

3. Results

3.1. Necropsy examination of the southern sea otter

Important microscopic lesions were present only in the CNS (Fig. 1A–E), and consisted of widely disseminated nonsuppurative meningoencephalomyelitis. Inflammation was most severe and diffuse in the cerebellar molecular layer and around blood vessels between the cerebellar folia, where macrophages and a few lymphocytes and plasma cells surrounded blood vessels. Severe gliosis and numerous small foci of acute necrosis were evident throughout the neuropil (Fig. 1A). Capillary endothelium was uniformly hypertrophied. Similar inflammation was present to a lesser degree throughout the midbrain, brainstem and gray matter in the upper cervical spinal cord, and as widely spaced foci in the cerebral cortex and white matter. Wherever inflammation was evident, it was accompanied by numerous *S. neurona*-like schizonts and merozoites (Fig. 1B–E). Microscopic lesions in other organs included acute pulmonary haemorrhage, lymphoid depletion and oedema in a tracheobronchial lymph node. Immature sarcocysts were seen in skeletal muscle fibers and rarely in cardiac muscle.

The schizonts and merozoites reacted with anti-*S. neurona* antiserum but not anti-*T. gondii* or *N. caninum* antiserum in the immunohistochemical tests (Fig. 1E). More parasites were seen in the sections reacted with anti-*S. neurona* antiserum that in the routine histological sections.

3.2. Isolation of S. neurona and development in vitro

Sarcocystis neurona was isolated in both BT and CV-1 cell cultures inoculated with brain homogenate. Development in BT and CV-1 cell cultures were similar and will be described together (Fig. 2A–F). Development was asynchronous and was by endopolyogeny.

Enlarging merozoites were present 1 day p.i. Enlarging merozoites and developing schizonts were present 2 days p.i. Mature schizonts with merozoites were first seen 3 days p.i. All developmental stages were present 4–6 days p.i. Merozoites in schizonts in BT cells were 5.4 ± 0.48 by 1.5 ± 0.29 µm (range, 4.0–5.6 by 0.8–1.6 µm; $N = 20$) and those in schizonts in CV-1 cells were 5.4 ± 0.38 by 1.4 ± 0.38 µm (range, 4.8–5.6 by 0.8–1.6 µm; $N = 20$) at 4 day p.i.

3.3. TEM

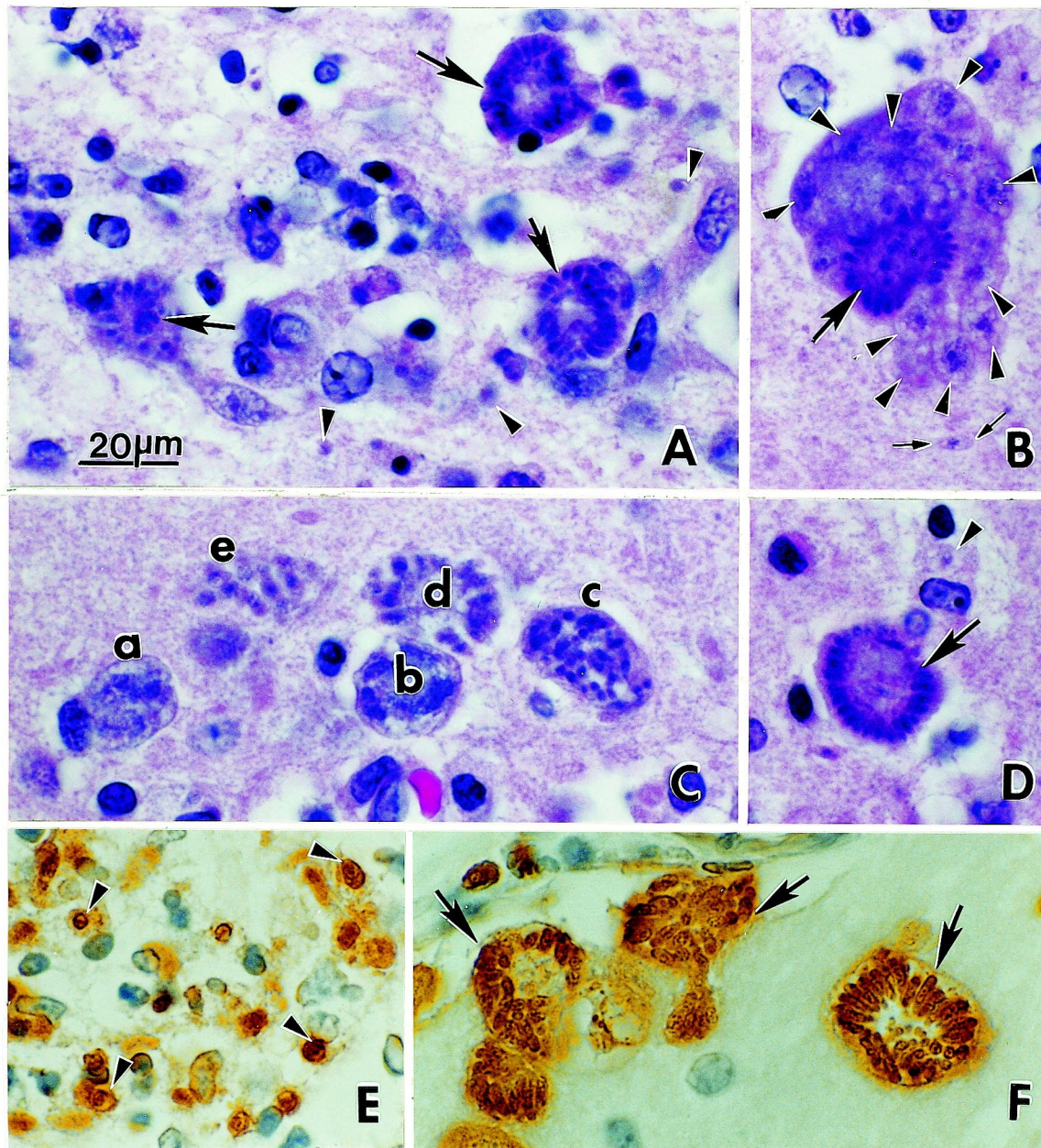Only asexual stages were observed in cell cultures. Development was by endopolyogeny and stages were

Fig. 1. Lesions and protozoa in sections of brain of a naturally infected Southern sea otter (A–E) and an INF-γ-KO mouse (F) inoculated with *Sarcocystis neurona* merozoites from the sea otter isolated from cell cultures. (A to D) = H&E stain, (E and F) immunohistochemical staining with rabbit anti-*S. neurona* antiserum. Bar = 20 µm and applies to all figures. (A) Necrosis of neuropil and infiltration by mononuclear cells. Note schizonts (arrows) and free merozoites (arrowheads). (B) A group of schizonts that appear to be in a neuron. Note at least nine are uninucleate (arrowheads) and a nearly mature schizont (arrow). Small opposing arrows point to a longitudinally cut free merozoite. (C) Five schizonts (a–e) in various states of development. Note the lobed nucleus in a and b. (D) Schizont (arrow) with peripherally arranged merozoites. Also note an intracellular merozoite (arrowhead). (E) Several individual merozoites in an area infiltrated by neutrophils. (F) Several mature schizonts (arrows).

located directly in the host cell cytoplasm. Merozoites contained a conoid, lacked rhoptries, had micronemes which extended one-fourth the way to the nucleus and contained other organelles typical for apicomplexan merozoites (Fig. 3A). Merozoites were observed attached to a residual body (Fig. 3B).

3.4. PCR and RFLP analysis

A 1100 bp PCR product was observed for reactions using DNA from merozoites from the Southern sea otter isolate, merozoites of *S. neurona* SN6 strain, and merozoites of *S. falcatula* SFVT-1-2 strain (Fig. 4).

The reaction products from the Southern sea otter isolate and *S. neurona* SN6 strain merozoites did not digest with *Hinf* I but were cut into 884 bp and 216 bp segments by *Dra* I (Fig. 4). The reaction product of from *S. falcatula* merozoites was cut into 745 bp and 355 bp segments by *Hinf* I but was not cut by *Dra* I (Fig. 4).

3.5. Mouse transmission studies

Clinical signs suggestive of encephalitis were noted 16 days p.i. in the INF-γ-KO mice. The signs consisted of slow movements, inactivity, paddling, and a
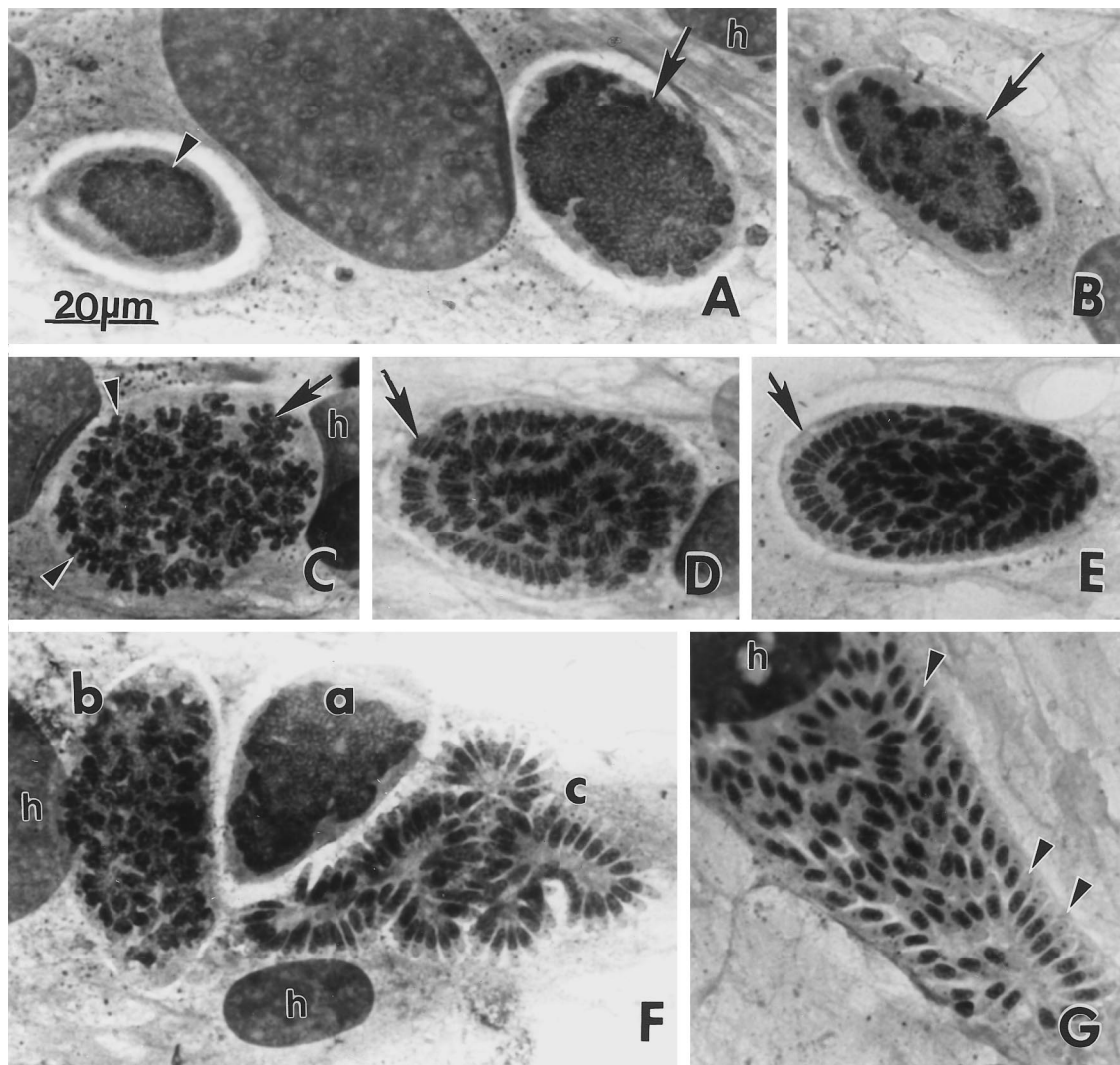

Fig. 2. Developmental stages of *Sarcocystis neurona* isolated from a Southern sea otter in bovine turbinate cell cultures. Giemsa type stain. Bar = 20 μm and applies to all figures. h = host cell nucleus in all figures. (A) Two uninucleate organisms, with undivided nucleus (arrowheads) and with developing nuclear lobes (arrow). (B) Schizont with about 30 nuclear lobes (arrow) arranged throughout the schizont. (C) Schizont with a highly lobed nucleus. The nucleus is divided into at least 28 sublobes and merozoites (arrow) are developing in association with these sublobes. (D) Schizont (arrow) with merozoites developing in packets. (E) Schizont (arrow) with merozoites forming centrally and peripherally. (F) Schizonts (a–d) in progressive states of development. Schizont (a) is immature with undifferentiated nuclear lobes (arrowhead). Schizont (b) is beginning merozoite formation. Schizont (c) has four groups of merozoites. (G) Schizont is mature and has completed development. Note merozoites (arrowheads) arranged in rows.

crouched appearance. All INF-γ-KO mice developed clinical disease and were sacrificed when it was judged that death was likely to occur in 1–2 days.

No sarcocysts were found in the INF-γ-KO mice. Microscopic lesions were confined to the brains of the INF-γ-KO mice. Lesions consisted of encephalitis associated with *S. neurona* schizonts and merozoites (Fig. 1F).

Serum antibody titres of 1:50 to *S. neurona* merozoites were observed in the IFAT for both mice examined. A 1:25 dilution of serum was not reactive to *S. falcatula* merozoites. No agglutinating antibodies to *T. gondii* or *N. caninum* were present at a 1:25 dilution of serum.

4. Discussion

The opossum is a definitive host for at least three species of *Sarcocystis* [2,16,17]. *Sarcocystis neurona* merozoites are infective for immunodeficient mice [2,18] while *Sarcocystis speeri* and *S. falcatula* merozoites are not infective for immunodeficient mice [16,17]. The infectivity of the merozoites from the Southern sea otter for INF-γ-KO mice is biological evidence that the parasite is *S. neurona*. This plus, the results of our PCR and restriction digestion with *Dra* I but not *Hinf* I indicate the parasite we have isolated is *S. neurona* [11].
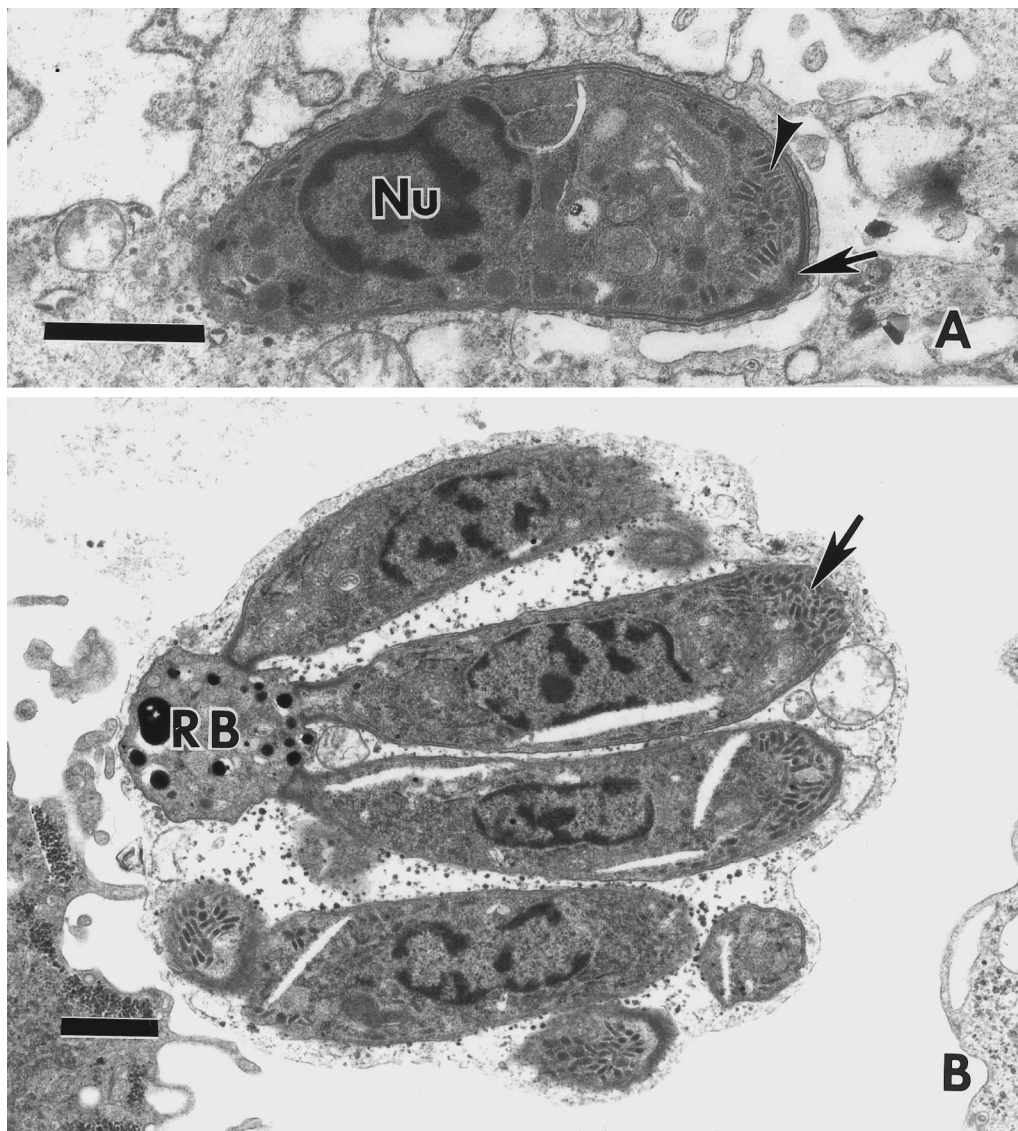

Fig. 3. Transmission electron micrographs of *Sarcocystis neurona* isolated from the Southern sea otter. (A) A merozoite demonstrating a nucleus (Nu), conoid (arrow), and micronemes (arrowhead). Note the micronemes are in the anterior one fourth of the merozoite. Bar = 1.0 μm. (B) Merozoites budding from a residual body (RB). Note the micronemes are in the anterior one-fourth of the merozoites (arrow). Bar = 1.0 μm.
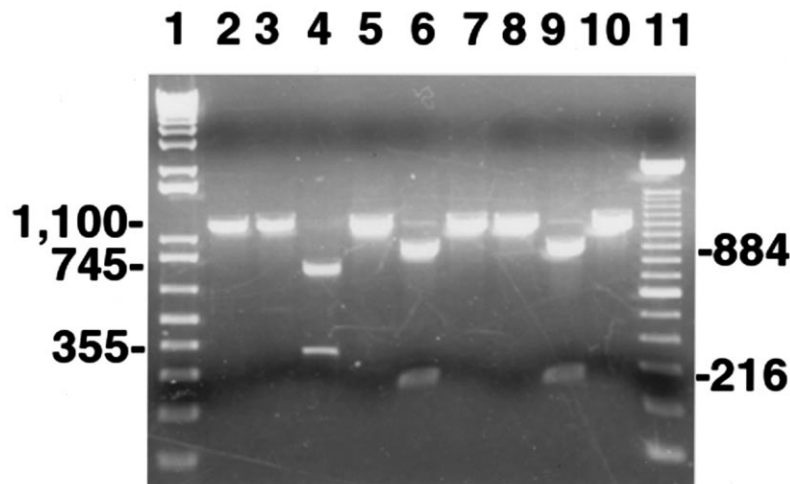
Fig. 4. Results of PCR using primer pairs JNB33/JNB54 and restriction enzyme digestion with *Dra* I and *Hinf* I of merozoite DNA from *Sarcocystis falcatula* VTSF-1-2 strain, *Sarcocystis neurona* from a Southern sea otter, and *Sarcocystis neurona* SN6 strain. Lane 1, high molecular mass marker, Lanes 2–4 *S. falcatula* VTSF-1-2 strain. 2 = PCR product, 3 = *Dra* I digestion, 4 = *Hinf* I digestion; Lanes 5–7 *S. neurona* from a Southern sea otter. 5 = PCR product, 6 = *Dra* I digestion, 7 = *Hinf* I digestion; Lanes 8–10 *S. neurona* SN6 strain, 8 = PCR product, 9 = *Dra* I digestion, 10 = *Hinf* I digestion; Lane 11 = low molecular mass marker.

The finding of immature sarcocysts in skeletal muscle is interesting. Sarcocysts are usually not seen in horses with *S. neurona* infection but unidentified sarcocysts have been seen in the other marine mammals [7,8].

Toxoplasmosis has been observed frequently in marine mammals [9,19–25]. Cole et al. [9] suggested that Southern sea otters acquired *T. gondii* infection by ingesting invertebrates, which were acting as phoretic agents for *T. gondii* oocysts. They believed that cat faeces containing *T. gondii* oocysts could be entering the marine environment through storm run-off and were picked up by invertebrate food items of the Southern sea otters. Buergelt and Bonde [19] believed that a Florida manatee became infected by drinking contaminated water. Oocysts of *Cryptosporidium* have been demonstrated in invertebrates from marine environments [26,27] demonstrating the ability of coccidial oocysts to get into these environments and to be obtained by potential phoretic agents. The oocysts of *Cryptosporidium parvum* can survive and still be infectious for at least 1 year in artificial seawater, and can be filtered out by invertebrates, retaining their infectivity in shellfish for up to 14 days [28].

Opossum faeces containing *S. neurona* sporocysts may enter the marine environment by storm run-off and be acquired by invertebrates which serve as phoretic agents. This is also the most likely way Southern sea otters and other marine mammals [7,8] become exposed to *S. neurona* sporocysts. It is theoretically possible therefore, that humans could become infected by ingesting shellfish or marine mammals which contain *Toxoplasma*, *Cryptosporidium*, or now, from the evidence presented here, *S. neurona*.

Acknowledgements

References

[1] Dubey JP, Davis SW, Speer CA, et al. *Sarcocystis neurona* n. sp. (Protozoa: Apicomplexa), the etiologic agent of equine protozoal myeloencephalitis. J Parasitol 1991;77:212–8.

[2] Dubey JP, Lindsay DS. Isolation in immunodeficient mice of *Sarcocystis neurona* from opossum (*Didelphis virginiana*) faeces, and its differentiation from *Sarcocystis falcatula*. Int J Parasitol 1998;28:1823–8.

[3] Stoffregen DA, Dubey JP. A *Sarcocystis* sp.-like protozoan and concurrent canine distemper virus infection associated with encephalitis in a racoon (*Procyon lotor*). J Wildl Dis 1991;27:688–92.

[4] Dubey JP, Hedstrom OR. Meningoencephalitis in mink associated with a *Sarcocystis neurona*-like organism. J Vet Diagn Invest 1993;5:467–71.

[5] Dubey JP, Hamir AN, Niezgoda M, Rupprecht CE. A *Sarcocystis neurona*-like organism associated with encephalitis in a striped skunk (*Mephitis mephitis*). J Parasitol 1996;82:172–4.

[6] Klumpp SA, Anderson DC, McClure HM, Dubey JP. Encephalomyelitis due to a *Sarcocystis neurona*-like protozoan in a rhesus monkey (*Macaca mulatta*) infected with simian immunodeficiency virus. Am J Trop Med Hyg 1994;51:332–8.

[7] Lapointe JM, Duignan PJ, Marsh AE, et al. Meningoencephalitis due to a *Sarcocystis neurona*-like proto-

zoan in Pacific harbor seals (*Phoca vitulina richardsi*). J Parasitol 1998;84:1184–9.

[8] Rosonke BJ, Brown SR, Tornquist SJ, et al. Encephalomyelitis associated with a *Sarcocystis neurona*-like organism in a sea otter. J Am Vet Med Assoc 1999;215:1839–42.

[9] Cole RA, Lindsay DS, Howe DK et al. Biological and molecular characterization of *Toxoplasma gondii* strains obtained from Southern sea otters (*Enhydra lutris nereis*). J Parasitol 2000;86: (in press).

[10] Lindsay DS, Dubey JP. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. Am J Vet Res 1989;50:1981–3.

[11] Tanhauser SM, Yowell CA, Cutler TJ, et al. Multiple DNA markers differentiate *Sarcocystis neurona* and *Sarcocystis falcatula*. J Parasitol 1999;85:221–8.

[12] Dubey JP, Mattson DE, Speer CA, et al. Characterization of a *Sarcocystis neurona* isolate (SN6) from a naturally infected horse from Oregon. J Euk Microbiol 1999;46:500–6.

[13] Lindsay DS, Dubey JP, Horton KM, Bowman DD. Development of *Sarcocystis falcatula* in cell cultures demonstrates that it is different from *Sarcocystis neurona*. Parasitology 1999;118:227–33.

[14] Dubey JP, Desmonts G. Serological responses of equids fed *Toxoplasma gondii* oocysts. Eq Vet J 1987;19:337–9.

[15] Romand S, Thulliez P, Dubey JP. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol Res 1997;84:50–3.

[16] Dubey JP, Lindsay DS. *Sarcocystis speeri* n. sp. (Protozoa: Sarcocystidae) from the opossum (*Didelphis virginiana*). J Parasitol 1999;85:903–9.

[17] Dubey JP, Speer CA, Lindsay DS. Isolation of a third species of *Sarcocystis* in immunodeficient mice fed feces from opossums (*Didelphis virginiana*) and its differentiation from *Sarcocystis falcatula* and *Sarcocystis neurona*. J Parasitol 1998;84:1158–64.

[18] Marsh AE, Barr BC, Lakritz J, Nordhausen R, Madigan RE, Conrad PA. Experimental infection of nude mice as a model for *Sarcocystis neurona*-associated encephalitis. Parasitol Res 1997;83:706–11.

[19] Buergelt CD, Bonde RK. Toxoplasmic meningoencephalitis in a West Indian manatee. J Am Vet Med Assoc 1983;183:1294–6.

[20] Inskeep W. 2nd, Gardiner CH, Harris RK, Dubey JP, Goldston RT. Toxoplasmosis in Atlantic bottle-nosed dolphins (*Tursiops truncatus*). J Wildl Dis 1990;26:377–82.

[21] Migaki G, Sawa TR, Dubey JP. Fatal disseminated toxoplasmosis in a spinner dolphin (*Stenella longirostris*). Vet Pathol 1990;27:463–4.

[22] Cruickshank JJ, Haines DM, Palmer NC, St. Aubin DJ. Cysts of a *Toxoplasma*-like organism in an Atlantic bottlenose dolphin. Can Vet J 1990;31:213–5.

[23] DiGuardo G, Agrimi U, Morelli L, Cardeti G, Terracciano G, Kennedy J. Post mortem investigations on cetaceans found stranded on the coasts of Italy between 1990 and 1993. Vet Rec 1995;136:439–42.

[24] Holshuh HJ, Sherrod AE, Taylor CR, Andrews BF, Howard EB. Toxoplasmosis in a feral northern fur seal. J Am Vet Med Assoc 1985;187:1229–30.

[25] Mikaelin I, Boisclair J, Dubey JP, Kennedy S, Martineau D. Toxoplasmosis in beluga whales (*Delphinapterus leucas*) from the St. Lawrence Estuary: Two case reports and a serological survey. J Comp Pathol 2000;122:73–6.

[26] Graczyk TK, Farley CA, Fayer R, Lewis EJ, Trout JM. Detection of *Cryptosporidium* oocysts and *Giardia* cysts in the tissues of eastern oysters (*Crassostrea virginica*) carrying principal oyster infectious diseases. J Parasitol 1998;84:1039–42.

[27] Fayer R, Lewis EJ, Trout JM, et al. *Cryptosporidium parvum* in oysters from commercial harvesting sites in the Chesapeake Bay. Emerg Infect Dis 1999;5:706–10.

[28] Tamburrini A, Pozio E. Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis*). Int J Parasitol 1999;29:711–5.